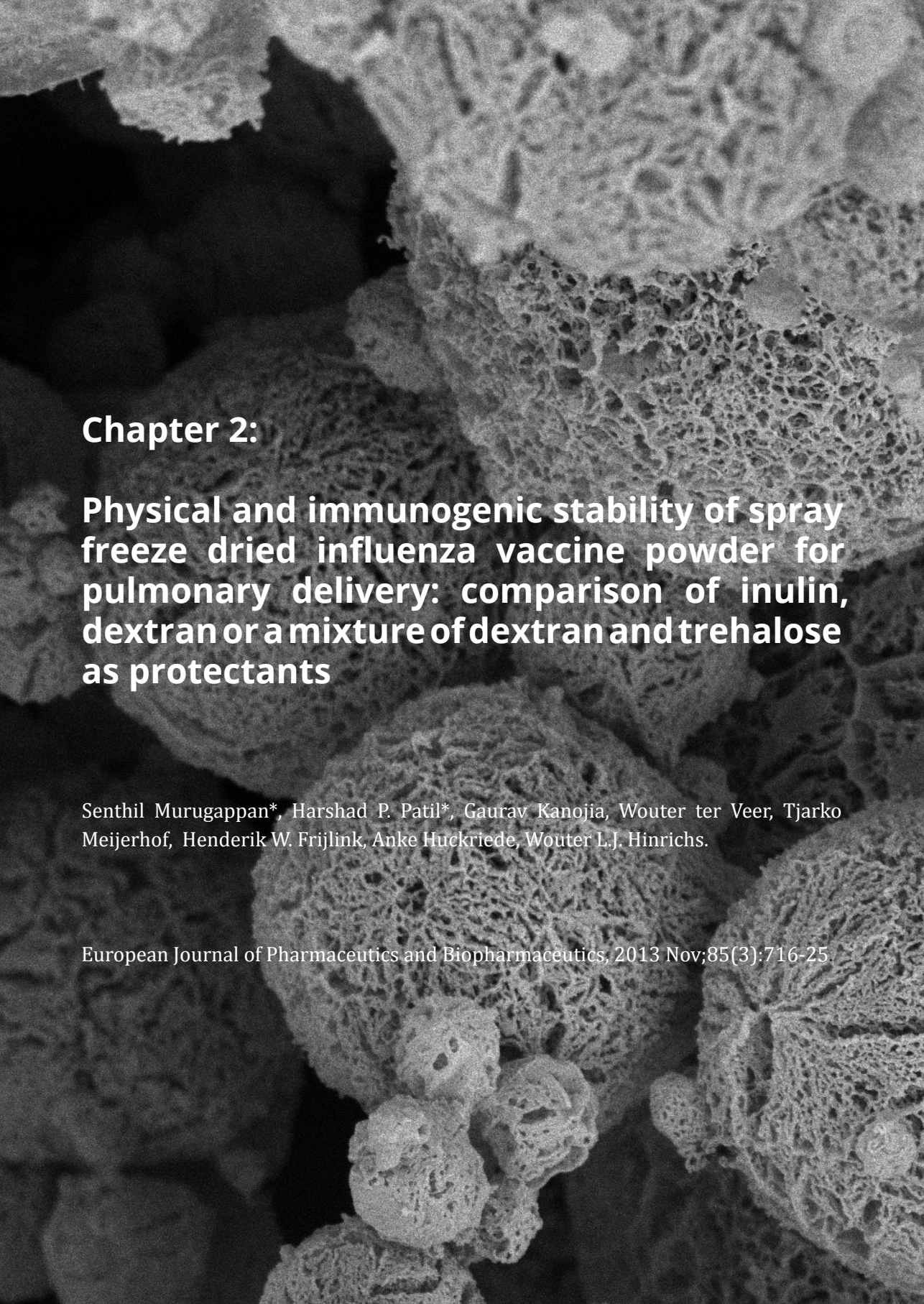
Development of adjuvanted influenza vaccines for pulmonary delivery

Patil, Harshad Padmanabh



Chapter 2:

Physical and immunogenic stability of spray freeze dried influenza vaccine powder for pulmonary delivery: comparison of inulin, dextran or a mixture of dextran and trehalose as protectants

Senthil Murugappan*, Harshad P. Patil*, Gaurav Kanojia, Wouter ter Veer, Tjarko Meijerhof, Henderik W. Frijlink, Anke Huckriede, Wouter L.J. Hinrichs.

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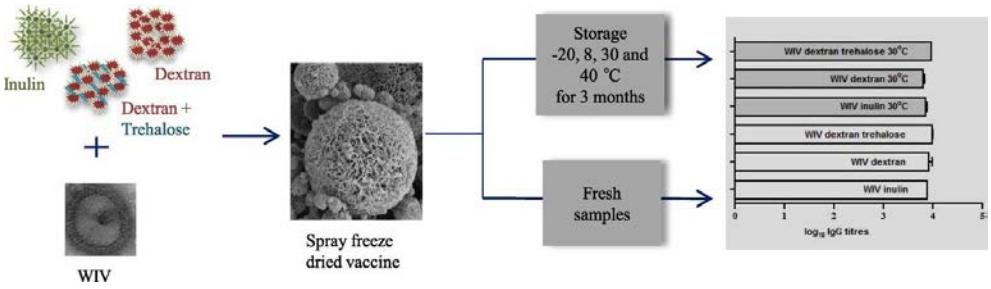
Abstract:

One of the advantages of dry influenza vaccines over conventional liquid influenza vaccines is that they can be used for alternative routes of administration. Previous studies showed that spray freeze drying is an excellent technique to prepare vaccine containing powders for pulmonary delivery (Amorij,J-P.; et al, 2007; Audouy,S.A.; et al, 2011). The aim of this study was to investigate the physical and immunogenic stability of spray freeze-dried whole inactivated virus influenza vaccine prepared by using inulin, dextran and a mixture of dextran and trehalose as protectants. Physical and biochemical characteristics of the vaccine powder were maintained at temperatures up to 30 °C for three months. In addition, *in vivo* data indicate that also the immunogenic properties of the vaccine were maintained under these storage conditions. On the other hand, *in vivo* results also revealed that subtle changes in powder characteristics were induced during storage at 30 °C. However, laser diffraction measurements showed that problems associated with these subtle changes can be overcome by using dry powder inhalers with an efficient powder dispersing capacity.

Graphical abstract:



Senthil Murugappan[a]*, Harshad P. Patil[b]*, Gaurav Kanojia[a], Wouter ter Veer[b], Tjarko Meijerhof[b], Henderik W. Frijlink[a], Anke Huckriede[b], Wouter L.J. Hinrichs[a]

a) Department of Pharmaceutical Technology and Biopharmacy, University of Groningen, Antonius Deusinglaan 1, 9713 AV, Groningen, The Netherlands.

b) Department of Medical Microbiology, Molecular Virology Section, University of Groningen, University Medical Center Groningen, Antonius Deusinglaan 1, 9713 AV, Groningen, The Netherlands.

*these authors contributed equally

1. Introduction

Influenza vaccination is the main strategy for the containment of the virus during influenza outbreaks. Unfortunately, every year many doses of influenza vaccines are discarded as the potency of the vaccine is lost due to the limited stability of the aqueous dispersion[1,2]. A major disadvantage of current influenza vaccines is that they remain stable within the narrow temperature range of 4 to 8 ºC only, which limits their widespread use especially during pandemic outbreaks. In addition, liquid vaccines are mainly administered via conventional routes like intramuscular (i.m.) and subcutaneous (s.c.) injection. Drawbacks like pain and needle fear reducing compliance, the chance for needle stick injuries and the need for trained health care workers to administer the injection, make injection a far from ideal method for vaccination [3]. Finally, influenza vaccines administered by injection predominantly induce a systemic immune response, which only provides protection at the systemic level and is not considered to be very effective in protecting the most vulnerable population, e.g. young children and elderly adults [4-6]. Although intranasally administered (i.n.) live attenuated influenza vaccine provides both local and systemic immune responses [7,8], it shares other drawbacks with conventional vaccines such as the requirement of a cold chain. Furthermore, this vaccine is not suitable for immune-compromised persons and children.

An attractive alternative to the current dosage forms could be a dry powder for pulmonary administration. Already more than 40 years ago several studies were performed to demonstrate the potential of pulmonary immunization using liquid influenza vaccine formulations [9,10]. The outcomes of these studies indicate that pulmonary immunization can induce same level of systemic immune responses compared to immune responses induced by vaccine administered via injection in humans. In addition, more recent studies show that pulmonary immunization also induce a local immune response, which bestows additional protection against influenza virus infection at the site of virus entry and might even provide cross-protection against infection by heterologous viruses [11]. A further advantage of pulmonary immunization is the improved patient compliance. In addition, these vaccines do not require the involvement of trained health care personnel as they can be self-administered. This procedural simplicity would be an advantage in particular during a pandemic.

In the early studies in man liquid vaccine formulations were used and they were administered via nebulizers which are currently known for their

poor and irreproducible dosing in the lung [12]. These problems may have been the reason for discontinuation of this research in the seventies of the previous century. However, modern technologies can overcome these problems. Moreover, scientific progress in drying technologies and dry powder inhalation systems has opened new possibilities for the development of stable dry powder inhalation systems for vaccines.

Pulmonary administration of influenza vaccines recently has regained interest because it became apparent that biopharmaceuticals such as vaccines can be brought in a dry and stable state by incorporating them in a matrix of a sugar glass by freeze drying, spray drying or spray freeze drying (SFD) [13-16]. In previous studies we have shown that various types of influenza vaccines can be stabilized by freeze drying using the oligosaccharide inulin as stabilizer [17-19]. For pulmonary delivery we envisage that whole inactivated virus (WIV) influenza vaccine would be the best option since it is more immunogenic than other types of influenza vaccines [20-23]. For an effective deposition in the lungs, WIV has to be formulated into powder particles with an aerodynamic particle size ranging from 1-5 μm [12,24,25], which can be achieved by SFD [26]. Recently, Audouy et al [27] showed that WIV can be SFD in the presence of inulin without loss of its immunogenicity and that the powder is suitable for pulmonary administration. However, neither the physical nor the immunogenic storage stability of the SFD WIV powder was investigated.

The aim of the present study was to investigate these aspects of storage stability for a period of three months at various temperatures. The following parameters were evaluated: (i) physical powder characteristics, (ii) biochemical integrity of WIV, and (iii) *in vivo* antigenicity after pulmonary administration to mice. Additionally, we studied the possibilities to replace inulin by dextran or a mixture of dextran and trehalose (dex/trh) as a stabilizer. Dextran has excellent amorphous bulking properties when lyophilized [28] and low molecular weight dextran can also act as a lyoprotectant [29]. Also trehalose, a disaccharide, can preserve biopharmaceuticals during lyophilization [30-32]. However, due to its large specific surface area, SFD trehalose rapidly absorbs moisture when exposed to air and due to its relatively low glass transition temperature (Tg) of 121 °C, the powder easily becomes sticky (personal observations). These properties of SFD trehalose will impose problems while handling and dispersing a powder to an aerosol for inhalation. Therefore, to circumvent problems with stickiness of SFD trehalose we investigated a mixture of trehalose with dextran as the latter possesses a much higher Tg (220 °C). Hence, dextran and dex/trh [30] were investigated next to inulin in this study.

2. Material and Methods

2.1. Virus

Live influenza virus A/Hiroshima/52/2005 (A/Hir/H3N2) was kindly provided by Solvay Biologicals (Weesp, The Netherlands).

2.2. Vaccine preparation

WIV was produced by inactivating live A/Hir/H3N2 virus by overnight incubation with 0.1 % β-propiolacton (Acros Organics, Geel, Belgium) at room temperature in citrate buffer (125 mM $Na_3C_6H_5O_7$, 150 mM NaCl, pH 8.2) under continuous rotation. The inactivated virus was then dialyzed overnight at 4 °C against hepes buffered saline (HBS, 2 mM hepes, 125 mM NaCl, 0.9 mM $CaCl_2.2H_2O$ and 0.5 mM $MgCl_2$; pH 7.4). WIV protein content was determined by micro-Lowry assay and its purity was analyzed by SDS-PAGE under reducing and non-reducing conditions followed by silver staining.

2.3. Spray freeze-drying

WIV was SFD together with inulin (4 kD; Sensus, Roosendal, The Netherlands) or dextran (6 kD; Sigma-Aldrich, Zwijndrecht, The Netherlands) or dex/trh (Cargill, Kerfeld, Germany) at a weight ratio of 1:1. A dispersion of WIV in HBS buffer containing 5 % w/v stabilizer was prepared at an HA:sugar weight ratio of 1:200. For placebo powder, a solution containing 5 % w/v stabilizer in HBS buffer without WIV was used. The dispersion/solution was pumped at a flow rate of 5 ml/min through a two-fluid nozzle (diameter 0.5 mm) of a Büchi 190 Mini Spray Dryer (Büchi, Flawil, Switzerland) and sprayed using an atomizing air flow of 600 l_n/hour in liquid nitrogen. The liquid nitrogen was allowed to evaporate after which the frozen droplets were placed on the shelf (pre-cooled to a temperature of -55 °C) of a Christ Epsilon 2-4 freeze dryer. Drying was performed at a pressure of 0.220 mBar with a condenser temperature of -85 °C. The shelf temperature was gradually increased from -55 °C to 4 °C over 32 hours. Thereafter, the pressure was decreased to 0.055 mBar and the shelf temperature was gradually increased to 20 °C over 11 hours. The powder vaccine was then collected in a hood at a relative humidity of 10 % or less and was stored at -20, 4, 30, and 40 °C in hermetically sealed injection vials for various periods of time.

2.4. Transmission electron microscopy

SFD vaccines were reconstituted with sterile water. Liquid and SFD formulations were dialyzed against ammonium acetate buffer (75 mM ammonium acetate, 2.5 mM Hepes, pH 7.4) overnight at 4 °C. Dialyzed samples were placed on a glow discharged 200 mesh copper grid covered with Formvar film. Samples were stained with 3% ammonium molybdate, pH 7.2 and analyzed on Philips CM 12 transmission electron microscope (TEM).

2.5. Hemagglutination assay

WIV containing 0.1 µg/µl of hemagglutinin (HA) was diluted 1:10 (w/v), in PBS (154 mM NaCl, 12 mM Na_2HPO_4 0.9 mM KH_2PO_4, pH 7.4) and 50 µl was added in 96-well V-bottom plates containing 50 µl of PBS and serially diluted twofold in PBS. Subsequently, 50 µl of 0.1% guinea pig red blood cells (RBC; Harlan, Zeist, The Netherlands) in PBS was added. Hemagglutination was determined two hours after incubation at room temperature. Hemagglutination titers were expressed as $\log_2$ of the highest dilution showing agglutination of RBC and recorded as one hemagglutination unit (HAU).

2.6. Physical characterization of the powders

Scanning electron microscopy (SEM) was performed with a JEOL JSM 6301-F microscope (JEOL Ltd., Tokyo, Japan). Samples were prepared by placing the powders on double-sided sticky carbon tape on a metal disk. Then the particles were coated with a layer of approximately 10 nm of gold using a Balzers 120B sputtering device (Balzer UNION, Liechtenstein). Images were captured at a magnification of 1000x.

The geometric particle size distribution of the SFD powders was measured using a HELOS compact model KA laser diffraction apparatus (Sympatec GmbH, Clausthal-Zellerfeld, Germany). The powders were dispersed using a RODOS dispersing system at a pressure of 1 bar or using the dry powder insufflator (Penn-Century Inc., Wyndmoor, USA).

The specific surface area of the SFD powders was determined using a Tristar surface analyzer (Micrometrics Instrument Corp., USA). The samples were loaded on the surface area analyzer, and the surface area was determined using the multipoint BET method from the nitrogen adsorption isotherm at 77K.

2.7. Immunization of mice

Animal experiment handling and work protocols were approved by the local animal welfare and use committee of the University of Groningen, The Netherlands. An *in vivo* study was carried out in 6-8 weeks old female BALB/c mice (Harlan, Zeist, The Netherlands).

Mice were immunized twice at an interval of three weeks with the different WIV formulations containing 5 µg HA. For pulmonary vaccination, mice were anesthetized by inhalation of isoflurane/O_2. Then the mice were intubated in vertical position with a modified Autograde catheter (Becton Dickinson, Breda, The Netherlands). SFD vaccine powder was delivered using a dry powder insufflator (Penn-Century Inc., Wyndmoor, USA). Approximately 1 mg of SFD vaccine powder was delivered to the lungs by giving three puffs with the insufflator. 50 µl of liquid aerosol vaccine was administered using an IA-1C Micro-sprayer aerosolizer for mice attached to the FMJ-250 high-pressure syringe (Penn-Century Inc., Wyndmoor, USA), and 50 µl of liquid aerosol of HBS was administered to the control group. Mice were placed in a recovery incubator at a temperature of 25 °C for two hours, and then placed back in the housing facility.

One week after the second dose, mice were sacrificed for evaluation of the immune response. After sacrifice, blood was withdrawn by heart puncture. Serum was stored at -20 °C until used. Nose washes and broncho-alveolar lavages (BAL) were obtained using 1 ml PBS, pH 7.4, containing complete protease inhibitor cocktail tablets (Roche, Almere, The Netherlands).

2.8. ELISA

Influenza specific IgG, IgG1, and IgG2a antibody amounts in serum and IgA antibody levels in nose wash, BAL and serum were measured using ELISA. The ELISA plates (Greiner bio-one, Alphen a/d Rijn, The Netherlands) were coated overnight at 37 °C with 500 ng/well of A/Hir/H3N2 WIV. Coated plates were washed once with coating buffer (17.8 mM Na_2CO_3, 22.5 mM $NaHCO_3$, pH 9.6) and blocked with 2.5 % milk powder in coating buffer. After washing once with coating buffer and twice with PBS containing 0.05 % Tween 20 (PBST, pH 7.2), serial dilutions of the serum samples were applied to plates. Plates were then incubated at 37 °C for 90 minutes and were subsequently washed with PBST. Then, 100 µl of horseradish peroxidase (HRP) conjugated antimouse IgG, anti-mouse IgG1, anti-mouse IgG2a or anti-mouse IgA (Southern Biotech, Birmingham, USA) diluted 1:5000 in PBST was added followed by incubation at 37 °C for

60 minutes for the detection of IgG, IgG1, IgG2a and IgA, respectively. After extensive washing, 100 µl phosphate-citrate buffer (0.2 M NaH_2PO_4, 0.1 M citric acid, pH 5, containing 0.04 % o-phenylenediamine and 0.012 % H_2O_2) was added. The enzymatic reaction was allowed to proceed at room temperature for 30 minutes and stopped by adding 50 µl of 2 M H_2SO_4. The absorbance was measured at 492 nm using a Synergy HT reader (BioTek, Winooski, USA). Average IgG titers were determined as $\log_{10}$ of the reciprocal of the sample dilution corresponding to an absorbance of 0.2 at a wavelength of 492 nm. IgA titers are presented as average of maximum absorbance of 1:1 diluted nose and lung washes. IgG1 and IgG2a concentrations were determined using a calibration curve made by overnight coating 0.1 µg anti mouse IgG at 37 °C. Following extensive washing, increasing concentrations of 100 µl of IgG1 or IgG2a (Southern Biotech, Birmingham, USA) was added to the plates. Average influenza HA-specific IgG1 or IgG2a responses are presented as concentrations µg/ml.

2.9. Hemagglutination inhibition (HI) assay

HI titers were determined by HI assay as described before [33]. In brief, 75 µl of serum (pooled per experimental group) was inactivated by incubation at 57 °C for 30 minutes. In order to prevent the non-specific hemagglutination, 225 µl of 25 % kaolin was added and incubated for 20 minutes. The suspension was then centrifuged for 2 min at 1400 x g. 50 µl of the resulting supernatant was added in duplicate to 96-well V-bottom plates containing 50 µl of PBS and, diluted serially till the last well of the plate. Subsequently, 50 µl of PBS containing 4 HAU was added to each well and mixed with a multichannel pipette. The plates were then incubated at room temperature for 40 minutes. Finally, 50 µl of 1 % guinea pig RBC was added and hemagglutination was allowed to proceed at room temperature for 2 h. The highest serum dilution capable of preventing hemagglutination was recorded as the HI titer.

2.10. Statistical analysis

The antibody titers are mentioned as geometric mean ± standard error mean. The difference in antibody titers was analyzed by one tailed Mann Whitney U-test at a confidence interval of 95 % ($P \leq 0.05$). The significance was denoted by an increase in the number of symbols: one symbol ($P \leq 0.05$); two symbols ($P \leq 0.01$).

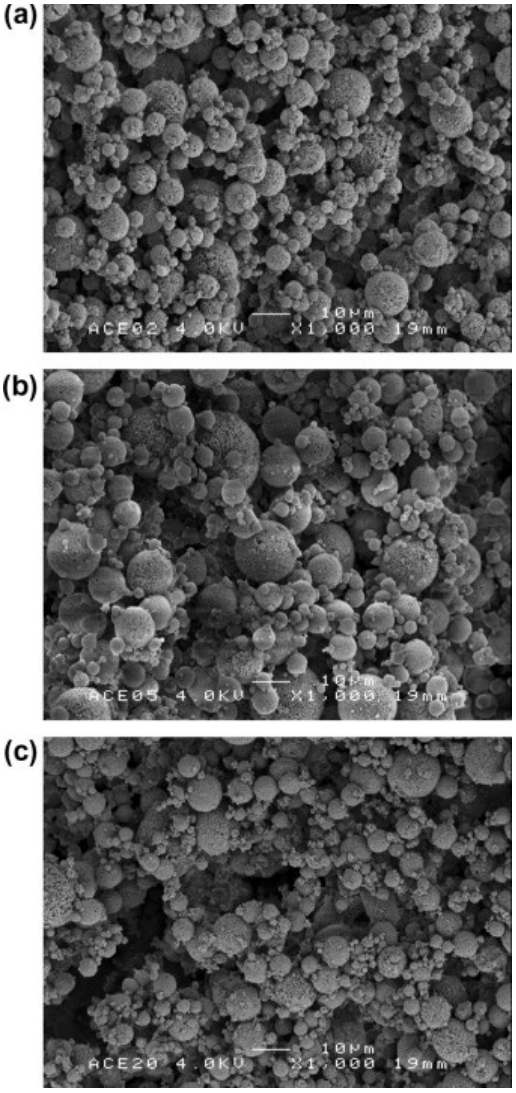


Figure 1. Analysis of powder morphology by SEM (a) SFD inulin (b) SFD dextran and (c) SFD dex/trh.

3. Results

3.1 Physical characterization of powders

Several physical characteristics of the SFD vaccine and placebo powders were evaluated in this study i.e. particle size, powder morphology, and specific surface area.

3.1.1. SEM of SFD powders

Analysis of the morphology of freshly prepared SFD inulin, dextran and dex/trh by SEM revealed that all three sugars formed highly porous spherical particles with interconnected pores and a particle size ranging from 1 to 10 µm (Fig 1). No difference in powder morphology was observed for the different sugars. The high porosity and interconnectivity of the pores can be contributed to the removal of the ice crystals by sublimation, which were formed during freezing. Hence, mirror images of ice crystals are seen in the SEM images.

3.1.2. Particle size of vaccine and placebo powders

To be deposited in the lungs after inhalation, a particle should have an aerodynamic particle size between 1 and 5 µm. Larger particles are likely to be deposited in the throat while smaller particles will be exhaled after inhalation [34]. Laser diffraction measurements revealed that SFD yielded powder particles with cumulative geometric size distribution of X_{10}, X_{50} and X_{90} of

3.5, 8 and 15 µm, respectively, (Table 1) which may seem too large. However, laser diffraction measurements yield the geometric particle size and not the aerodynamic particle size. The aerodynamic particle size can be calculated from the geometric particle size by the following equation [35]:

$$d_{ae} = d_e \sqrt{\frac{\rho_p}{\rho_o \chi}}$$

Where, d_{ae} is the aerodynamic diameter, d_e the geometric particle size, ρ_p the density of the particles (g/cm^3), ρ_0 the unit density (1 g/cm^3) and, χ the dynamic shape factor. The atomized liquid droplet leaving the nozzle was measured by the laser diffraction and had an average geometric particle size of approximately 8 µm that was similar to the particle diameter of SFD powders. Apparently, the particle size of liquid droplet did not shrink during freeze-drying. Therefore, the solid content of the solution before SFD can be used to calculate the density of the final SFD powder particles. Since the total solid concentration of the solution before SFD was 50 mg/ml, the density of the SFD powder particles will be: ρ_p = 0.05 g/cm^3. The dynamic shape factor of spherical particles is 1 and the SEM (Fig 1) confirmed the spherical shape of particles. Hence, it can be calculated that the aerodynamic diameter of X_{10}, X_{50} and X_{90} of the SFD vaccine powder were around 0.8, 1.8 and 3.3 µm, respectively, indicating that except for a small fraction these powders were suitable for inhalation.

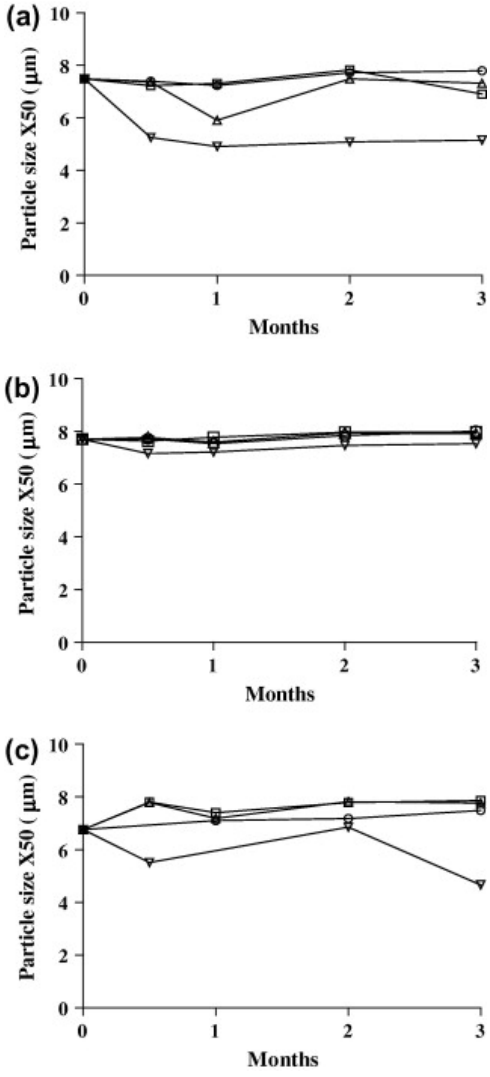


Figure 2. Particle size analysis of SFD (a) inulin, (b) dextran, and (c) dex/trh for a period of 3 month at -20, 2-8, 30, and 40 °C. Values are represented as X50. The particle size was measured in triplicate. Since the standard deviations were small the error bars are not visible.

Laser diffraction measurements revealed that immediately after production the geometric particle size distributions of the SFD inulin, dextran and dex/trh powders containing WIV and the corresponding placebo powders were similar (Table 1). Since for physical characterization huge amounts of powder are required and because the sugars were in large excess in the powders (weight ratio HA/sugar = 1/200), placebo powders were used to evaluate the effects of storage on the shape and size characteristics.

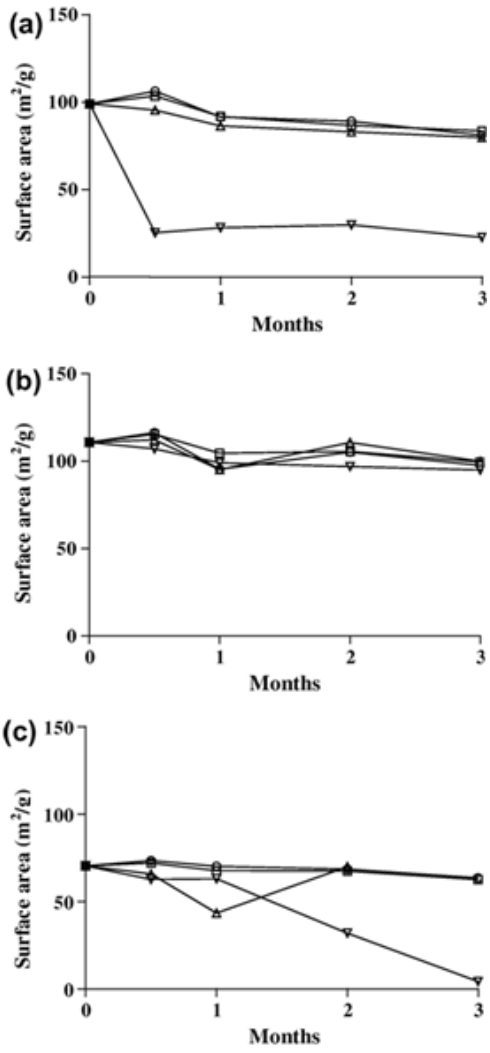


Figure 3. Specific surface area analysis of SFD (a) inulin, (b) dextran and, (c) dex/trhstored for a period of 3 month at -20, 2-8, 30, and 40 °C The specific surface area was measured in triplicate. Since the standard deviations were small the error bars are not visible.

To evaluate whether the particle size of the SFD powders changed during storage, the powders were analyzed by laser diffraction at various time intervals. The measurements (Fig. 2) revealed that the particle size of SFD inulin, dextran and dex/trh stored at -20, 4 or 30 °C did not change. However, the particle size of SFD inulin stored at 40 °C was reduced 15 day after storage. The particle size of the SFD dextran was found to remain the same for 3 months at 40 °C. In case of the SFD dex/trh stored at 40 °C, particle size slightly reduced after 15 days of storage, and substantially after 3 months of storage.

3.1.3. Evaluation of dry powder insufflator performance

The powder dispersing capacity of the dry powder insufflator was evaluated with the SFD inulin, dextran and dex/trh powders using laser diffraction. It was found that the particle size distribution of powder leaving the dry powder insufflator was highly irreproducible between the puffs. Moreover, it was found that

the dry powder insufflator was dispersing particles with a bimodal distribution, i.e. particles with a size of about 10 µm and some with a size of about 100 µm. The X_{10}, X_{50} and X_{90} values of a typical measurement with SFD powders dispersed by the insufflator are shown in Table 2. Laser diffraction measurements using the RODOS disperser showed no bimodal particle size distribution but small particles only (average of around 8 µm; see Table 1). Dry powder insufflator has been reported efficient in dispersing spray dried powders [36]. However, our results show that the dry powder insufflator was not able to deagglomerate the highly fluffy SFD powders efficiently.

3.1.4. Specific surface area of placebo SFD powders

The specific surface area of placebo SFD powders before and after storage at different temperatures up to three months was evaluated by BET analysis (Fig. 3). This analysis indicated that, immediately after preparation, the produced powders, irrespective of the sugar used, had a high surface area, thus confirming the presence of highly porous particles as observed by SEM. Yet, SFD inulin and SFD dextran both had a higher specific surface area (100-120 m^2/g) than dex/trh (around 75 m^2/g). As mentioned in section 1.2., the density of the SFD particles were the same for all three sugars, which implies that the porosity was same in all cases. Since the specific surface area of SFD inulin and dextran was smaller than that of SFD dex/trh, it can be concluded that the pore sizes in the SFD inulin and dextran particles were smaller than those in the SFD dex/treh particles. This indicates that during the freezing step of the SFD process, nucleation of ice crystals was slower in the inulin and dextran solution than in the dex/trh solution which might be related to the lower viscosity of the latter.

The specific surface area of all SFD powders remained unchanged during storage for 3 months at -20, 4 or 30 °C. However, at 40 °C the specific surface area of the SFD inulin was significantly reduced after 15 days of storage. Also the specific surface area of the SFD dex/trh was not stable during storage at 40 °C as it was reduced to 40 m^2/g after 2 months and to 5 m^2/g after 3 months. In contrast, the specific surface area of the SFD dextran sample was maintained during storage at 40 °C for 3 months.

In conclusion, all three SFD powders could be stored at temperatures up to 30 °C for at least 3 months without substantial change of their physical powder characteristics. However, higher storage temperatures led to changes in the physical properties of SFD inulin and dex/trh but the powder properties were retained in SFD dextran.

Table 1. Comparison of geometric diameter of spray freeze-dried vaccine and placebo powder as determined by laser diffraction using the RODOS disperser.

Sample	Vaccine powder (µm±SD)			Placebo powder (µm±SD)		
	X_{10}	X_{50}	X_{90}	X_{10}	X_{50}	X_{90}
Inulin	3.56 ± 0.01	8.01 ± 0.03	14.84 ± 0.02	2.94 ± 0.12	7.48 ± 0.39	20.93 ± 0.11
Dextran	3.67 ± 0.02	8.30 ± 0.04	15.42 ± 0.01	2.29 ± 0.12	7.64 ± 0.05	17.95 ± 0.17
Dex/trh	3.25 ± 0.01	7.65 ± 0.31	14.78 ± 0.23	2.78 ± 0.83	6.96 ± 0.12	14.85 ± 0.33

Table 2. Results of a typical example of a laser diffraction measurement of SFD inulin, dextran and mixture of dextran and trehalose using the dry powder insufflator.

Sample	**Freshly prepared powder (µm)**		
	X_{10}	X_{50}	X_{90}
Inulin	21.99	100.55	153.13
Dextran	19.81	92.04	149.55
Dex/trh	4.06	13.96	119.84

3.2. Characterization

3.2.1. Morphology of WIV

The immunogenicity of WIV relies on the presence of intact virus particles, which retain the structure of the live virus and harbor all virus components including the single stranded viral RNA which is an important trigger of innate immune reactions [37]. To evaluate whether the particulate nature of WIV was preserved during the drying process, the SFD powders were reconstituted and then analyzed by TEM. (Fig. 4).

TEM images of liquid WIV and WIV SFD in the presence of inulin, dextran or dex/trh and then reconstituted with water revealed that stress related to SFD did not affect the particulate nature of WIV. Presence of WIV particles with sizes of 100 nm to 150 nm and clearly visible spikes on the viral membrane indicates that inulin, dextran and dex/trh are suitable stabilizers during SFD of WIV.

3.2.2. Hemagglutination titers

The biological activity of HA after SFD with inulin, dextran or dex/trh and subsequent storage at -20, 4, 30 or 40 °C for up to three months was evaluated by measuring virus binding to a target cell membrane in a hemagglutination assay (Fig. 5).

The activity of HA in unprocessed WIV (Fig 5a) was reduced by more than 100-fold (7 (log_2)) when stored at 30 °C or 40 °C for 1 month. In addition, there was a gradual loss in hemagglutination titer for samples stored at -20 and 4 °C. The hemagglutination titers of WIV after SFD with inulin, dextran or dex/trh were found to be unchanged after storage for a period of 3 months at temperatures up to 40 °C (Fig 5b-d).

To further determine the molecular stability of the proteins present in WIV, freshly prepared vaccines and vaccines stored for three months at 30 °C were analyzed by SDS-PAGE. The gel of freshly prepared WIV showed a band pattern consistent with the structural proteins present in WIV (Fig. 5e). Four bands associated with proteins of WIV were observed on the gel, viz., HA1: 47 kD, NA: 50 kD and HA2: 29 kD. No difference was found in the band pattern between freshly prepared liquid or reconstituted SFD formulations and SFD formulations stored at 30 °C. Yet, the band color intensity was observed to be lower for the SFD formulations stored at 30 °C. However, a larger difference was observed in the banding pattern between freshly prepared WIV and liquid WIV stored at 30 °C. Bands of HA1 and HA2 which could be easily identified in freshly prepared WIV were almost absent in WIV stored at 30 °C for 3 months.

Preservation of the hemagglutinating function of HA and the unchanged protein patterns revealed by SDS-PAGE demonstrate that the stresses of the SFD process and storage of the SFD powders at 30 °C for three months did not result in aggregation or cleavage of the WIV proteins.

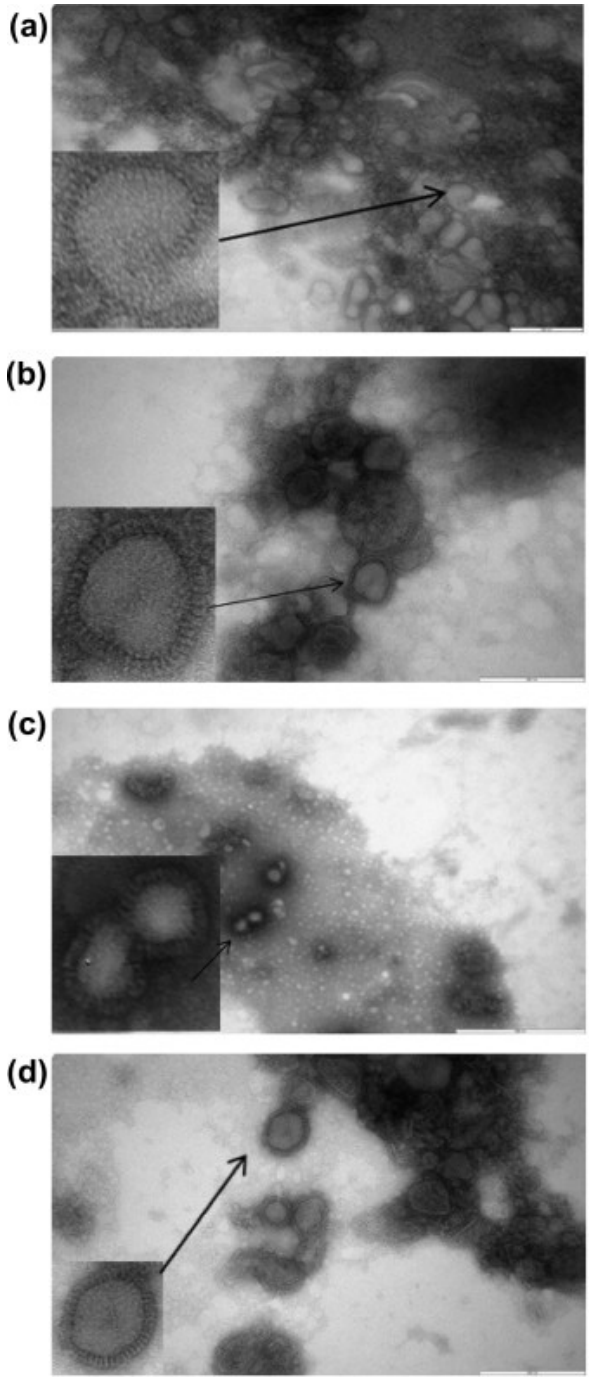


Figure 4. TEM images of (a) unprocessed WIV and WIV SFD in the presence of (b) inulin, (c) dextran and (d) dex/trhand then reconstituted.

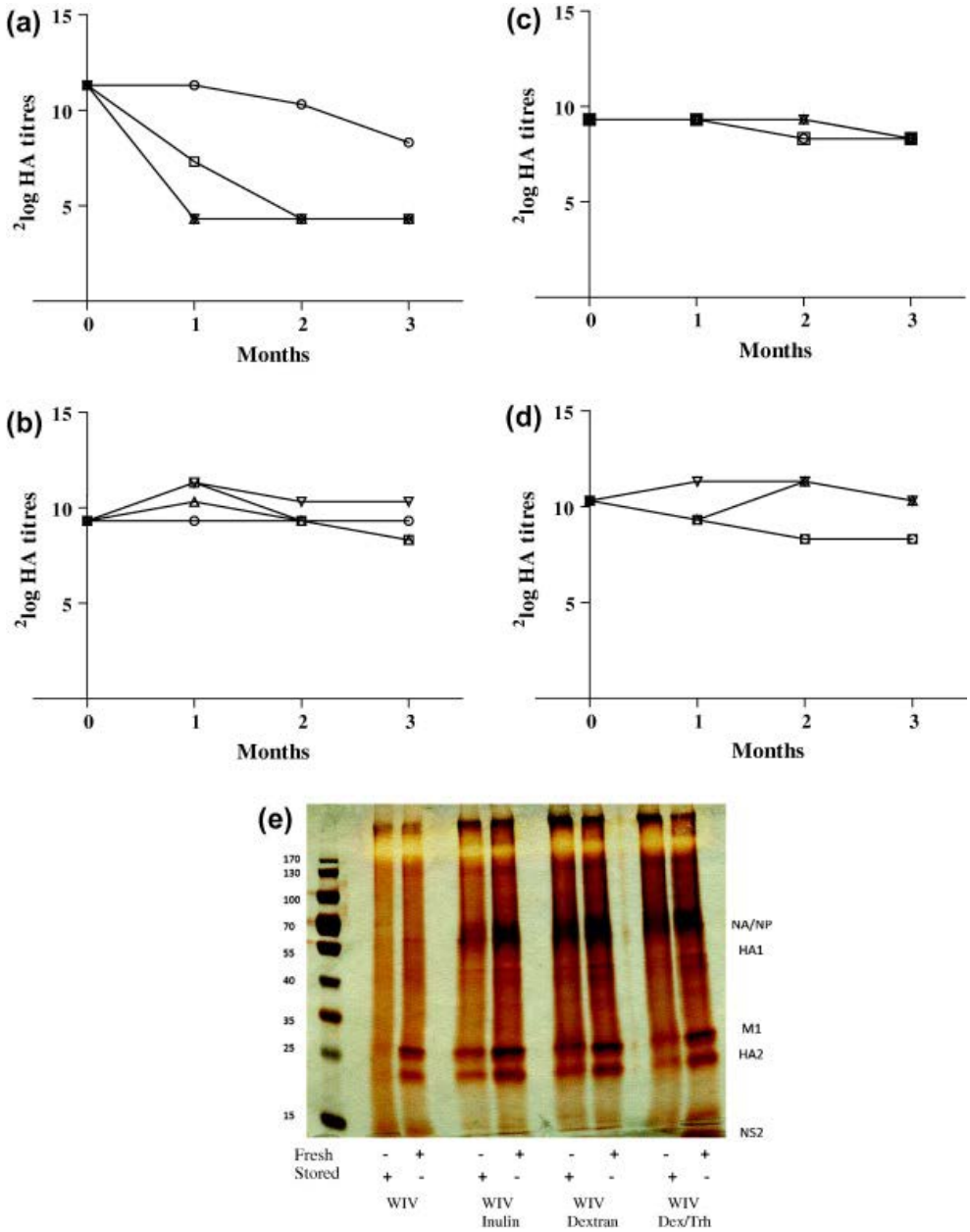


Figure 5. Hemagglutination titers of WIV in (a) unprocessed liquid, (b) SFD inulin, (c) SFD dextran and (d) SFD dex/trh stored for 3 months at -⊖- -20, -⊟- 2-8, -△- 30, and -▽- 40 °C. The HA titers of SFD dex/trh stored at 30 °C are superimposed with HA titers of samples stored at 40 °C from 2 months. The HI titers were measured in triplicate. Since no differences in HA titers were found no error bars are shown. (e) SDS PAGE analysis of freshly prepared and SFD and reconstituted WIV stored for three months at 30 °C.

3.3. Immune response after vaccination

3.3.1. Evaluation of humoral immune responses evoked by SFD and reconstituted vaccines

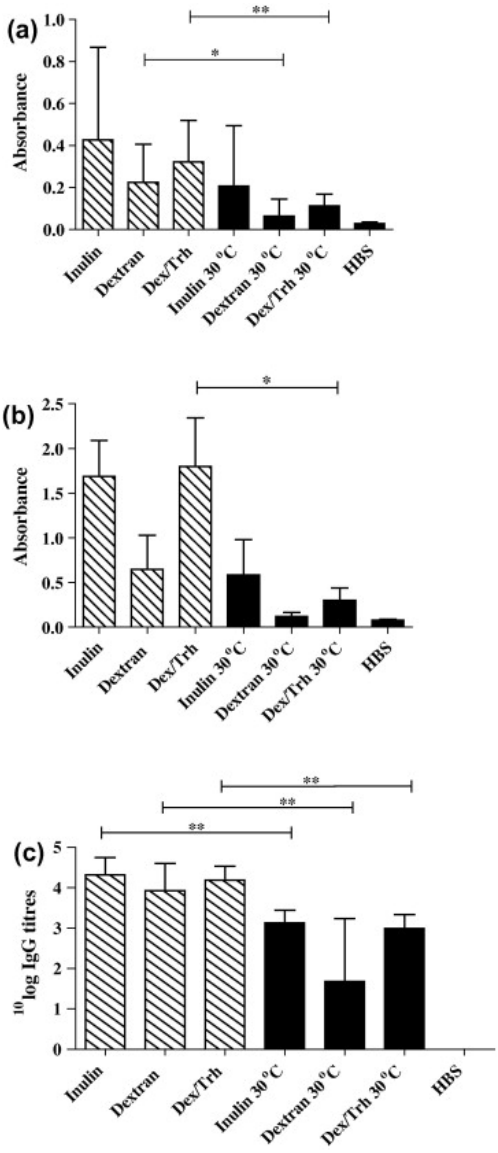


Figure 6. WIV specific humoral immune responses induced in mice two weeks after booster dose by pulmonary immunization of SFD vaccine powders. (a) Nasal IgA (b)BAL IgA and (c) serum IgG antibody responses induced by freshly prepared (striped bars) and stored vaccines at 30 °C (black bars).

To evaluate the effect of the different sugars on preservation of the immunogenic properties of the vaccines mice were immunized twice via the pulmonary route with the different SFD vaccine powders which were either freshly prepared or stored at 30 °C for 3 months. Nose washes, BAL and serum samples were collected one week after the second dose and analyzed by ELISA (Fig 6a-c). No significant differences were found in the immune responses evoked by freshly prepared SFD vaccine powders with the different sugars except for BAL IgA which was somewhat lower for the SFD vaccine powder based on dextran. Comparison of IgA antibody responses in nose and BAL washes between mice that received freshly prepared and stored SFD vaccine powders showed that in all cases there was a decreased IgA antibody production in the mice vaccinated with the stored formulations although the differences were not always significant (Fig 6a-b). Similarly, evaluation of IgG antibody titers in serum revealed that SFD vaccine powders stored at 30 °C for three months induced significantly lower IgG antibody titers than freshly prepared SFD formulations vaccine powders (Fig 6c), and the same trend was seen for the HI titers of the sera (data not shown).

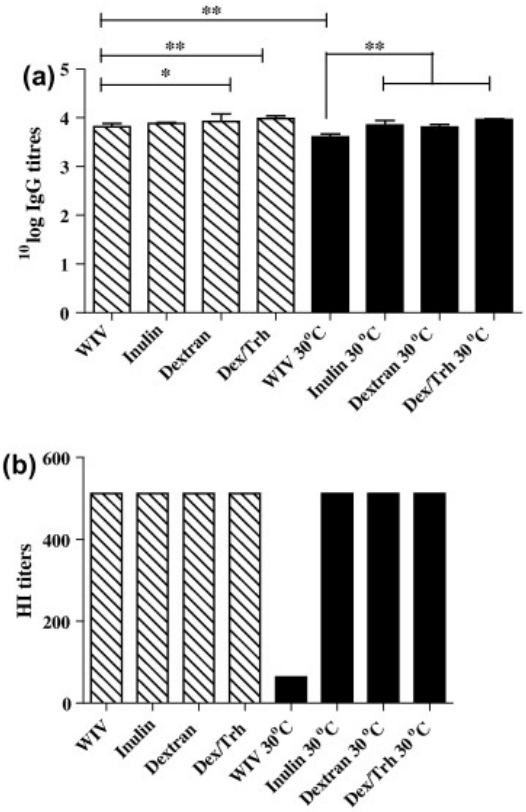


Figure 7. WIV specific humoral immune responses evoked by reconstituted SFD vaccines. (a) Serum IgG antibody responses and (b) Serum HI titers induced by freshly prepared (striped bars) and stored vaccines at 30 °C (black bars) administered in liquid form in mice.

The lower mucosal and systemic antibody induction in mice after pulmonary administration of SFD vaccine powders stored at 30 °C could be due to loss of antigenicity of the vaccine or due to changes in physical powder characteristics after storage leading to less effective vaccine administration or due to both. However, our in vitro studies showed that both the physical powder characteristics and the integrity of the vaccine did not change substantially during storage. To elucidate this inconsistency, freshly prepared as well as stored SFD vaccine powders were reconstituted with water and then pulmonary administered to mice with the micro-sprayer. The serum IgG antibody response as determined by ELISA (Fig 7a) showed that stored liquid WIV induced significantly lower levels of IgG antibody titers in mice than freshly prepared WIV or SFD vaccine powders reconstituted after storage. Furthermore, SFD vaccine powders, reconstituted after storage, induced a similar level of IgG antibody titers as SDF vaccine powders reconstituted immediately after preparation. HI titers were in line with the IgG antibody titers as the stored liquid WIV elicited significantly decreased HI titers compared to SFD powders reconstituted immediately after preparation or after storage or the freshly prepared liquid formulation (Fig 7b).

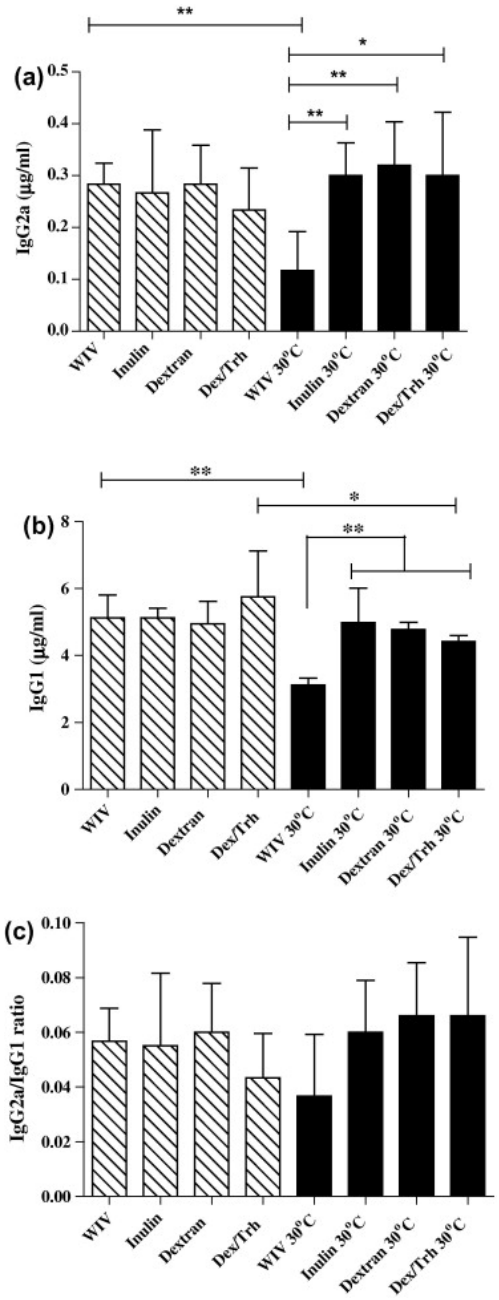


Figure 8. The phenotype of immune response induced in mice by reconstituted SFD vaccines determined by IgG subtypes analysis. Serum (a) IgG2a (b) IgG1 and (c) IgG2a/IgG1 antibody ratios induced by freshly prepared vaccines (striped bars) and stored vaccines at 30 °C (black bars).

The subtypes of IgG antibodies (IgG2a and IgG1) induced after pulmonary administration of stored (three months at 30 °C) or freshly prepared, liquid WIV or reconstituted SFD vaccine powders were analyzed by ELISA. It was found that stored liquid WIV induced significantly lower amounts of IgG2a and IgG1 antibodies than freshly prepared or stored SFD vaccine powders or freshly prepared WIV (fig 8a-b). Furthermore, storage of the SFD vaccine powders did not affect the induction of IgG1 and IgG2a antibody titers. Analysis of the IgG2a/IgG1 ratio (Fig. 8c) shows that pulmonary vaccination induced a IgG1-dominated antibody response which is in line with a previous study [27].

Overall, the HI titers, IgG titers and the IgG2a and IgG1subtype titers demonstrate that the antigenicity of WIV stored at 30 °C for three months was better preserved in SFD than in liquid vaccine formulation. Furthermore, the stabilizers used for SFD had no effect on the preservation of the immunogenicity of the vaccines.

3. Discussion and conclusion

In this study, we prepared a dry powder influenza WIV formulation by SFD using inulin, dextran, or dex/trh as stabilizing excipients. The aerodynamic diameter of the SFD powders was about 0.8, 1.8 and 3.3 μm for X_{10}, X_{50} and X_{90} indicating that almost all of the powder particles were suitable for inhalation. Furthermore, we showed that for all three stabilizers the SFD vaccine powders could be prepared without loss of the particulate nature, biochemical integrity and receptor-binding property of the incorporated WIV as shown by TEM, SDS-PAGE and HA assay, respectively.

During storage of the SFD powders for three months at temperatures up to 30 °C, the physical powder characteristics, i.e. particle size distribution and specific surface area, remained the same. However, when stored at 40 °C, the size and specific surface area of SFD particles of inulin and dex/trh were reduced. These results indicate that the inulin and dex/trh particles shrunk during storage, yet without displaying macroscopic viscous flow. These observation can be related to the Tg of the powders. The Tg of dry dextran was found to be 220 °C, whereas the Tg of dry inulin and dry dex/trh was in both cases 154 °C. All these Tg values are far above storage temperatures implying that all three sugars are suitable as stabilizers. However, it is to be realized that residual moisture is a potent plasticizer for sugar glasses. Thus, residual moisture present in the samples (although not determined) will strongly reduce the Tg [38]. As all powders were prepared by an identical procedure it can be assumed that all contained equal amounts of residual moisture. For inulin and dex/trh this moisture might have been sufficient to reduce the real Tg values from 154 °C to close to 40 ºC. In contrast, for dextran with a high initial Tg of 220 °C, the residual moisture was possibly insufficient to lower the Tg to 40 ºC. The shrinkage of the inulin and dex/trh containing particles is in line with this reasoning. The shrinkage indicated that there was some translational molecular mobility but not to such an extent that macroscopic viscous flow occurred.

Furthermore, WIV incorporated in the SFD powders retained its particulate nature, biochemical integrity and receptor-binding properties during three months storage at temperatures up to 30 °C. As expected, the unprocessed liquid WIV formulation was less stable. Replacing inulin by dextran and dex/trh revealed that both these sugars are also excellent stabilizers for WIV during SFD and subsequent storage. Additionally, WIV incorporated in dextran was even stable at temperatures as high as 40 °C. Overall, WIV SFD in the presence of all three sugars yielded a stable product when stored at 30 °C. Hence, the

SFD vaccine powders stored at 30 °C for three months were selected for the immunization of mice. The pulmonary administration of the SFD vaccine powders induced IgA in the nose and BAL washes, serum IgG and serum HI titers. However, to our surprise the stored SFD vaccine powders were found to elicit significantly lower antibody and HI titers than the freshly prepared SFD vaccine powders. In contrast, immunization experiments with reconstituted SFD vaccines demonstrated that the immunogenicity of WIV was not decreased during storage of the SFD powders. This indicates that although not detected by laser diffraction and BET measurements, the physical properties of the SFD vaccine powders must have changed during storage such that delivery to the lungs was hampered. Possibly, the results can be explained as follows: for the determination of the particle size distribution by laser diffraction, the RODOS disperser was used. It is well known that the RODOS dispenser is a very powerful disperser [39]. In contrast, the dry powder insufflator was found to exhibit very poor dispersing capacities for the SFD powders as not only primary particles left the insufflator but also large agglomerates. Therefore, it could be that during storage subtle changes to the physical properties of the SFD powder occurred that were not significant enough to be measured by BET and laser diffraction using the RODOS, but caused the poor delivery of the SFD powders into the lungs of the mice by the dry powder insufflator. However, in a clinical situation, powder delivery can be improved by using inhalers like the Novolizer® or Twincer®, which disperse powders better than the dry powder insufflator used in this study [40-43].

In previous studies, we demonstrated that various influenza vaccines can be stabilized by incorporating them in inulin matrices which is in agreement with the present study [18,19,27,33]. Additionally, our results demonstrate that dextran and dex/trh can stabilize WIV equally well. Consequently, it is expected that vaccine powders prepared with either of these sugars can be safely stored at room temperature for extended period. The stabilized and powdered influenza vaccines can overcome the limitations imposed on liquid vaccines by cold chain requirements. Furthermore, the possibility of self-administration makes vaccination campaigns independent of the availability of trained health care personnel and may improve compliance with vaccination recommendations in high-risk populations.

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